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THE BINDING OF THYROID HORMONES TO PHOSPHOLIPID MEMBRANES

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SUMMARY

1. Thyroxine and tri-iodothyronine are bound by liposomes (bimolecular phospholipid membranes produced by dispersing egg-yolk lecithin in water). At pH 7·4 the 'partition coefficient' between water and the phospholipid is $1{\cdot}2 \times 10^4$ for thyroxine and $2{\cdot}2 \times 10^4$ for tri-iodothyronine. The partition coefficient falls off rapidly in more alkaline solutions as the phenolic group on the hormones becomes ionized.

2. The binding of thyroxine by membranes is very rapid, occurring at rates equivalent to those found in the binding of thyroxine by serum.

3. Lecithin membranes are readily permeable to thyroxine (the oil:water partition coefficient is 1·7).

4. The binding of thyroid hormones by perfused rat hearts and the binding of thyroid hormones by liposomes are similar in their sensitivity to pH. Simple alcoholic extracts of tissues have strong thyroxine-binding activity.

5. These results are discussed in relation to the nature and function of the tissue thyroxine-binding reaction.

INTRODUCTION

Tissues accumulate large amounts of thyroxine and tri-iodothyronine when perfused with saline media (Hillier, 1968, 1969*b*). The nature of this binding process, however, is unknown although Tata (1964) has postulated the existence of specific cellular thyroxine-binding proteins, analogous to the binding proteins in plasma. In this paper it is demonstrated that thyroid hormones become concentrated at phospholipid membranes and it is suggested that most of the hormone in tissues is trapped in this way.

Lecithin, when shaken with water, disperses into microscopic spherical globules called liposomes. Each one is composed of a series of bimolecular membrane spheres packed one inside the other, each membrane being separated from the next by a water layer. Hand-shaken lecithin may

contain between one and twenty membranes. When dispersed further by ultrasonic vibration (sonication) they break down to single compartment spheres containing only one membrane. These structures have been successfully used as models for cells and mitochondria, their surfaces showing the basic properties of unit membrane (Bangham, 1968).

METHODS

Resin uptake experiments. It was the purpose in these experiments to measure the rate at which thyroid hormones were taken up from solution by Amberlite resin (IRA 400). The radioactive solutions were pumped through columns of resin at a fixed rate and the amount of the hormone taken up at various time intervals was determined by cutting the columns into appropriate lengths and measuring their radioactivity. Basically two types of experiment were performed. In one group (illustrated in Figs. 2 and 4) thyroxine was added to the lecithin suspensions at least an hour before they were perfused through the resin columns. In the other experiments (illustrated in Fig. 3) a pulse injection of thyroxine was made into columns of resin while they were being perfused with suspensions of liposomes. The experimental procedure was exactly the same as that used in a previous study (Hillier, 1970) except that wider tubing was used (diameter 5 mm) to give flow rates of 1 cm/sec (for the experiments illustrated in Fig. 3 flow rates up to 3 cm/sec were employed and the columns were frozen solid before being cut into sections). Fairly coarse resin (14–52 mesh) was used and since the liposomes were less than 5 μ in diameter they were not mechanically trapped by the columns. However, to avoid any contamination from this source the columns were vigorously perfused with saline for a minute or so at the end of the experiments; the perfusates during these times became completely clear showing that they were free of lecithin. All experiments were performed at room temperature.

Equilibrium dialysis. Two ml. suspensions of phospholipid, containing radioactive hormone, were enclosed in 10 cm long sacs of Visking dialysis tubing ($\frac{18}{32}$ in.). The sacs were gently shaken for 4 hr in 10 ml. buffer in a plastic pot. One ml. aliquots from the inside and the outside of the sacs were then counted to give the concentration of the total and of the free hormone respectively. Control experiments showed that 4 hr was more than adequate for equilibration to take place. All experiments were performed at room temperature.

Experiments with perfused hearts. The methods used have been described in detail elsewhere (Hillier, 1968). The solutions used for perfusion were buffered with 0·02 M phosphate buffer and not bicarbonate; and they were passed through a fine scintered glass filter just before use. All experiments were performed at room temperature.

The partition coefficient for thyroxine between olive oil (B.P.) and 0·1 M phosphate buffer pH 7·4 was determined by shaking up equal volumes (10 ml.) of the two solvents (thyroxine being present initially in the aqueous phase) for 12 hr, separating the phases by centrifugation and counting aliquots of each

The suspensions of phospholipid in 0·1 M phosphate buffer were prepared by vigorously shaking (by hand) about 200 mg of the material in about 5 ml. water and then diluting to final strength with buffer. The lecithin used was L-α-lecithin, IIE (Sigma) and it contained lysolecithin and sphingomyelin as impurities. In some experiments sonicated liposomes were used; 0·5 ml. water containing 50 mg lecithin was exposed to high intensity sound (80 kc/sec) for half an hour. This was sufficient to disperse the material into mainly single compartment liposomes. This change can be followed macroscopically since the normally turbid suspension becomes clear and

translucent. Sonication was performed at the Agricultural Research Council Institute of Animal Physiology, Babraham. The treatment given to the lecithin suspensions is there routinely found to produce mainly single compartment liposomes (Johnson & Bangham, 1969) although in this particular case no confirmation was attempted by electron microscopy. It is certain, however, from the clearness of the suspension produced, that the sonicated liposomes were very much smaller and much less complex than the ones produced by hand shaking.

The maximum pH obtainable with the phosphate buffer system used was about 8·8 and in some experiments it was necessary to extend this range; this was done by adding small amounts of sodium hydroxide. The pH of the solutions obtained was stable throughout the various procedures employed.

Lipid extracts of liver. Rats were anaesthetized and their livers thoroughly perfused with 100 ml. 0·9 % NaCl (Hillier, 1969*a*). A gram of the tissue was then homogenized in water to give a final concentration of 100 mg liver/ml. Aliquots of this homogenate were diluted with 0·1 M phosphate buffer and then used for equilibrium dialysis. Further 1 ml. aliquots of the homogenate were extracted with 15 ml. 98 % alcohol for 10 min at 90° C. The mixture was centrifuged and the alcohol decanted off. The extract was evaporated to dryness in air (initially at 90° C and later at room temperature) and the residue resuspended to give the required concentration for equilibrium dialysis.

Iodide contamination. The solutions of radioactive hormone were contaminated with 2–5 % inorganic radio-iodide. In control experiments it was found that this iodide was rapidly taken up by Amberlite resin, freely permeable in the dialysis system, not bound by liposomes, not concentrated by rat hearts and not soluble in olive oil. In each experiment, therefore, the iodide contamination was estimated chromatographically (Hillier, 1968) and the results appropriately corrected.

Radioactive materials. [^{125}I]L-thyroxine, [^{125}I]-3-5-3′-L-tri-iodothyronine and inorganic ^{131}I were obtained from The Radiochemical Centre, Amersham.

RESULTS

The binding of thyroid hormones by liposomes

Equilibrium dialysis experiments. Liposomes are much too large to penetrate Visking dialysis membranes whereas free thyroxine can. This difference was used to estimate, in suspensions of liposomes, the proportion of the total thyroxine that was present in free solution. The hormone was allowed to partition between two compartments separated by a dialysis membrane, egg-yolk lecithin being present in only one compartment at a concentration of 1 mg/ml. in 0·1 M phosphate buffer. After equilibration the thyroxine concentration on the side containing only buffer gave the free thyroxine concentration and on the side containing phospholipid gave the concentration of both the free and the bound hormone. The results are illustrated in Fig. 1. They show that only a fraction of the hormone was present in a free state, the remainder being bound by the liposomes. There was a marked difference between thyroxine and tri-iodothyronine. For both hormones the proportion present in the free state increased as the pH was raised but with the curve for tri-iodothyronine being displaced some

1·5–2·0 pH units to the right. The pK for the phenolic group on thyroxine is 6·5 and for tri-iodothyronine is 8·4; the sharp fall-off in binding of the hormones may therefore be associated with the development of strong charge on this group (see Discussion). It was calculated that, on a weight for weight basis, the 'partition coefficient' between the water and the phospholipid at pH 7·4, was $1{\cdot}2 \times 10^4$ for thyroxine and $2{\cdot}2 \times 10^4$ for tri-iodothyronine. The use of a partition coefficient here is convenient but not

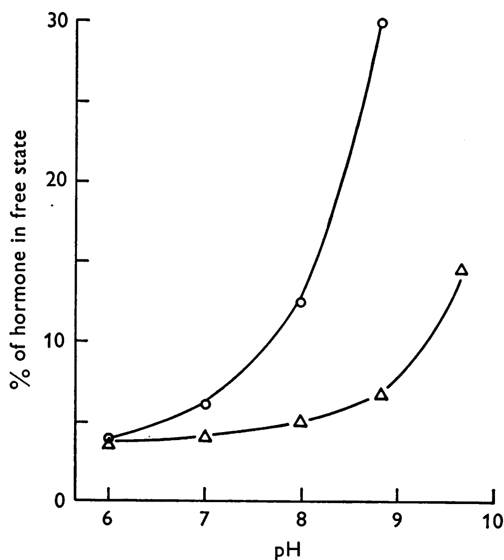


Fig. 1. The percentage of thyroxine (○) and tri-iodothyronine (△) present in the free state in suspensions of phospholipid (1 mg/ml.) in 0·1 M phosphate buffer at various pH. Each point represents the mean of two experiments. Hormone concentration 1×10^{-3} μg/ml.

strictly applicable since it implies simple solution of the hormone into the phospholipid phase, in fact the binding reaction is almost certainly more specific than this. In these experiments the hormone concentration was 1×10^{-3} μg/ml. but the same results were given with 1 μg/ml. At this higher concentration the ratio of thyroxine to lecithin molecules in the liposomes was about 1:500.

Resin uptake experiments. The vigorous concentration of thyroid hormones by phospholipid membranes was examined by a different method. It has been shown that columns of Amberlite resin rapidly and firmly bind thyroid hormones present in free solution (Hillier, 1970) and in further control experiments it was shown that the rate of this process was unaffected by variations in pH between 6 and 9. Liposomes are not trapped

by ion exchange resins and so it would be expected that, if thyroid hormones are strongly bound by phospholipid membranes, then the rate of hormone uptake by resin ought to be reduced by the presence of liposomes. This effect was investigated in some fifty or more experiments. It was found that liposomes did in fact impede the trapping of thyroid hormones by Amberlite resin and that the effect was very sensitive to pH. The results of experiments at pH 8·8 are illustrated in Fig. 2. At pH 6 uptake was slow,

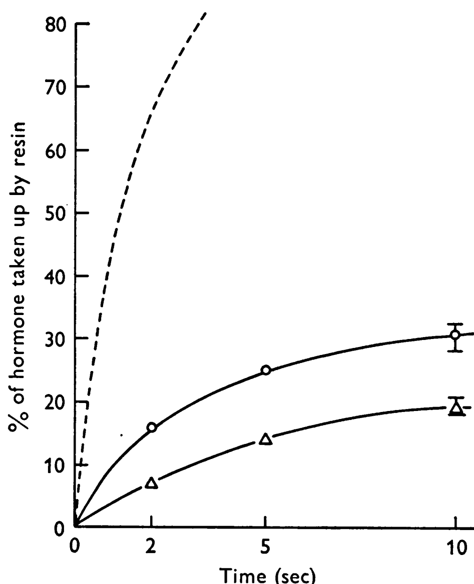


Fig. 2. The uptake of thyroxine (○) and tri-iodothyronine (△) by Amberlite resin from 0·1 M phosphate buffer pH 8·0 containing 1 mg lecithin/ml. Each curve shows the mean of four experiments and the vertical lines indicate the range of the individual observations. The dashed line shows the uptake of both hormones in the absence of lecithin. Hormone concentration 1×10^{-3} μg/ml.

with some 12–15 % of both hormones being captured by the resin within 10 sec; at pH 8·8 the uptake of tri-iodothyronine was slightly faster, about 23 % being captured in 10 sec but for thyroxine at this higher pH there was a much faster uptake rate with about 48 % captured. In fact, the 'strength' of the hormone-phospholipid interaction at various pH, shown by these resin uptake studies, was exactly similar to that shown by the experiments with equilibrium dialysis illustrated in Fig. 1. Again the results were independent of the hormone concentration between 1×10^{-4} and 1 μg/ml.

It would be expected, at least over the initial few seconds, that the rate

of thyroxine uptake by the resin would be proportional to the concentration of thyroxine present in free solution. By comparing the uptake rate from ordinary buffer and from liposome suspensions therefore, an approximate estimate could be obtained of the percentage of free thyroxine present in suspensions of phospholipid. For suspensions containing 1 mg lecithin/ml. 0·1 M phosphate buffer at pH 7·4, the percentage of free thyroxine was estimated at 11 % and of free tri-iodothyroxine at 7 %, values similar to those obtained by equilibrium dialysis.

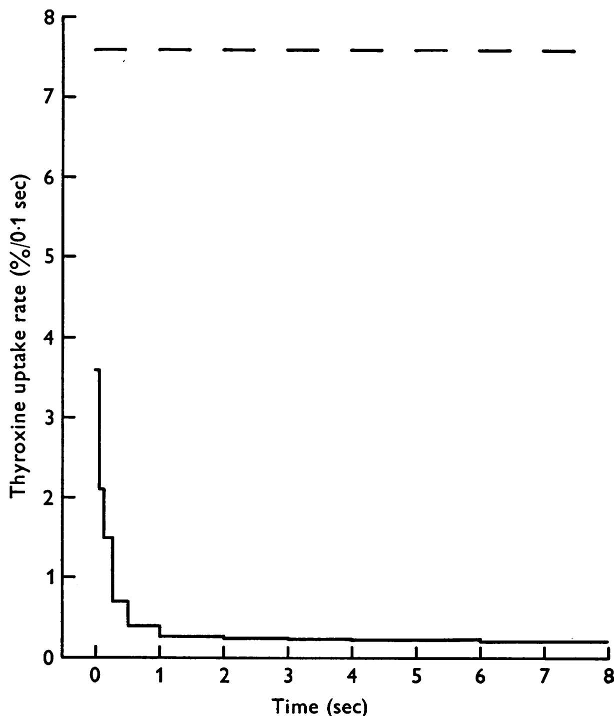


Fig. 3. A tracer dose of thyroxine was injected into a resin column perfused with 0·1 M phosphate buffer pH 6·0 containing 10 mg lecithin/ml. The thyroxine uptake rate (the amount of thyroxine taken up per 0·1 second expressed as a percentage of the amount still present in solution) is plotted against the time after the injection was made. The dashed line shows the uptake rate for free thyroxine (in the absence of lecithin). Hormone concentration 1×10^{-3} μg/ml.

The rate of thyroxine binding by liposomes. Free thyroxine injected into a resin column perfused with phosphate buffer is taken up exponentially with a $t_{\frac{1}{2}}$ of about 1·2 sec. This value depends on how closely packed the columns are and in this study wide rather loosely packed columns were

used. The rate of thyroxine uptake plotted in Fig. 3 is the amount of thyroxine taken up per 0·1 sec expressed as a percentage of the amount of thyroxine still present in solution at the beginning of the time interval. For free thyroxine injected into phosphate buffer at pH 6 this value was between 7 and 8 % per 0·1 second and remained constant with time since the uptake was an exponential process. For free thyroxine injected into buffer containing lecithin at a concentration of 10 mg/ml. the uptake pattern was very different. Five experiments were performed and a typical result is illustrated in Fig. 3. Even within the first 100 msec of mixing the uptake rate was only about half of the rate expected for free thyroxine. This indicated that the thyroxine–lecithin interaction was very rapid, occurring at rates similar to those found in the binding of thyroxine to serum (Hillier, 1970).

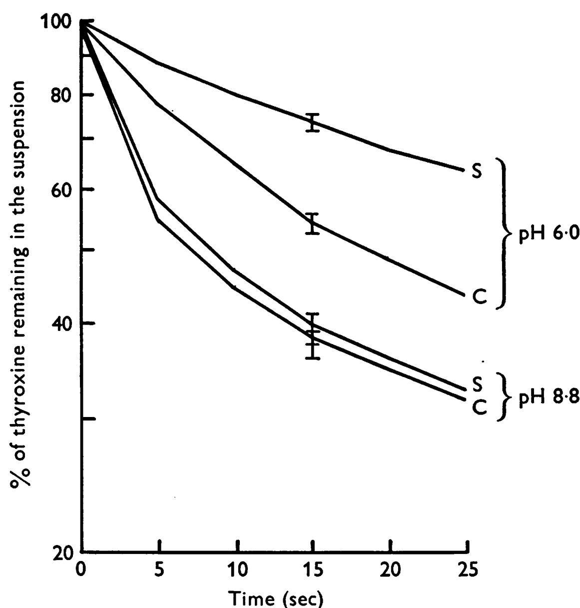


Fig. 4. Suspensions of sonicated (S) and non-sonicated (C) lecithin (1 mg/ml.) containing thyroxine were perfused through resin columns at pH 6·0 or 8·8 and the percentage of the hormone still remaining in the suspension was measured at various time intervals. There were three experiments in each group and the vertical lines indicate the range of individual observations. Hormone concentration 1×10^{-3} μg/ml.

The permeability of lecithin membranes to thyroxine. Handshaken liposomes may contain between 1 and 20 membrane spheres. Thyroxine, given sufficient time, would bind to each one so that the majority of the bound hormone would be separated from the free aqueous phase by several mem-

branes, each of which would have to be crossed before it could be trapped by the resin. Sonicated liposomes contain only one or two membranes and most of the bound thyroxine here could be released directly and become immediately available for capture by the resin. Comparing the speeds with which resin captures thyroxine from ordinary and from sonicated liposomes gives therefore an indication of the permeability of the membranes to the hormone. Fig. 4 shows the results of such an experiment. Lecithin suspensions (1 mg/ml.) were equilibrated with thyroxine for at least 3 hr and then perfused through resin columns. At pH 8·8 thyroxine was released from single and multi-compartment liposomes at almost the same rate showing that the membranes were offering little barrier to thyroxine diffusion. At pH 6, where the hormone binds much more strongly to the phospholipid, the release from the sonicated liposomes was a little faster but even here the membranes were offering little resistance to diffusion. The ability of substances to penetrate lipid membranes is usually associated with a high oil:water partition coefficient ($> 0{\cdot}1$) and in an experiment the partition coefficient for thyroxine between olive oil and phosphate buffer at pH 7·4 was estimated at 1·7 (mean of four observations). Further, it was found that the proportion of free thyroxine in suspensions of phospholipid was not dependent on whether the material was hand-shaken or sonicated. This would be expected of course if the membranes were permeable to the hormone.

Hydrogen ion concentration at the membrane surface. The complex array of phospholipids present in material prepared from natural sources contains a net excess of acidic head-groups. The membranes derived from them are therefore negatively charged over a wide range of pH. This causes the hydrogen ion concentration at the membrane surface to be substantially higher than it is in the bulk aqueous phase (Bangham, 1968). During the course of this investigation an observation was made which allowed an estimation of the surface pH. The indicator bromothymol blue was found to bind to the micelles. Equilibrium dialysis showed that at a phospholipid concentration of 5 mg/ml., more than 90% of the indicator was attached to the membranes. On the other hand it was poorly soluble in olive oil and it was concluded that the indicator was adsorbed to the membrane surface. When the bulk aqueous phase was buffered to pH 8·8 the indicator on the membranes had the yellow-green colour characteristic of pH 7·2 and with the bulk phase at pH 7·4 the pH of the membrane surface was 6·0. These results suggested that over the neutral pH range the surface pH was about 1·5 units lower than in the bulk phase.

Experiments with alcoholic extracts of liver

In these experiments simple hot alcoholic extracts of liver were prepared, the alcohol was evaporated off and the lipid residue resuspended in phosphate buffer. The ability of these extracts to bind thyroid hormones was then examined by equilibrium dialysis. Unfortunately the procedure was difficult to make quantitative (especially with resuspension of the

dried extract) and further, it was assumed that the extraction procedure did not influence the ability of the lipids to bind the hormones. The results therefore must be regarded as essentially qualitative. Three sorts of experiment were performed.

First, the binding power of lipid extracts was compared with that of the tissue homogenates from which they were prepared. In a group of three experiments liver homogenates were examined containing 5 mg tissue/ml. phosphate buffer at pH 7·4. In these suspensions 12–15 % of the thyroxine was present in the free state. In suspensions of the alcoholic extracts at the same equivalent concentration the proportion of free thyroxine was 22–27 %. These results suggested that at least half of the thyroxine binding activity in the homogenates was due to lipid material.

In a second experiment a comparison was made between thyroxine and tri-iodothyronine. For lipid extracts (equivalent to 10 mg liver/ml.) at pH 6, the proportion of free thyroxine was 5·5 % (mean of two experiments) and of free tri-iodothyronine 7·4 %. At pH 8·8 the value for tri-iodothyronine was slightly higher at 9·5 % and for thyroxine it was substantially increased to 31 %. These results were very similar to those obtained with egg-yolk lecithin (Fig. 1).

In a third experiment it was found that boiling the homogenates for 5 min did not impair their ability to bind thyroxine. Specific binding proteins might be expected to denature under these conditions whereas phospholipids being relatively thermostable would be little affected. Control experiments confirmed this latter point.

Experiments with perfused rat hearts

It has previously been demonstrated that, at physiological pH, rat hearts bind more tri-iodothyronine than thyroxine (Hillier, 1968, 1969*b*); this is also true of liposomes and lipid extracts of liver. If a large proportion of the hormones were simply binding to the lipid membranes in the tissue then it would be expected that the uptake process would show a sensitivity to pH similar to that shown by the thyroxine-phospholipid interaction (i.e. a marked difference in uptake between the two hormones at pH 8·8 but with little difference at pH 6, the uptake of thyroxine being especially sensitive to pH). The uptake process on the alternative hypothesis (trapping by specific binding proteins) would probably have a different pH sensitivity, though not necessarily so.

In a group of five experiments rat hearts were perfused with 0·9 % NaCl for 90 min to wash out any plasma proteins in the interstitial fluid (Hillier, 1969) and then further perfused with Krebs–Ringer phosphate solution at either pH 6 or pH 8·8, each solution containing radioactive thyroxine at a concentration of 1×10^{-3} μg/ml. The effect on thyroxine uptake of

switching from one pH to the other was examined and a typical result is illustrated in Fig. 5. It shows that thyroxine uptake was very sensitive to pH, being very much faster at pH 6.

In another experiment rat hearts were perfused initially with 0·9% NaCl for 30 min and then further perfused for an hour with Krebs–Ringer phosphate solution at pH 6·0 or 8·8 containing either thyroxine or tri-iodothyronine at a concentration of 1×10^{-3} μg/ml. Table 1 shows the amount of hormone taken up after 60 min perfusion. As with liposomes the binding of thyroxine by the hearts was much more sensitive to pH

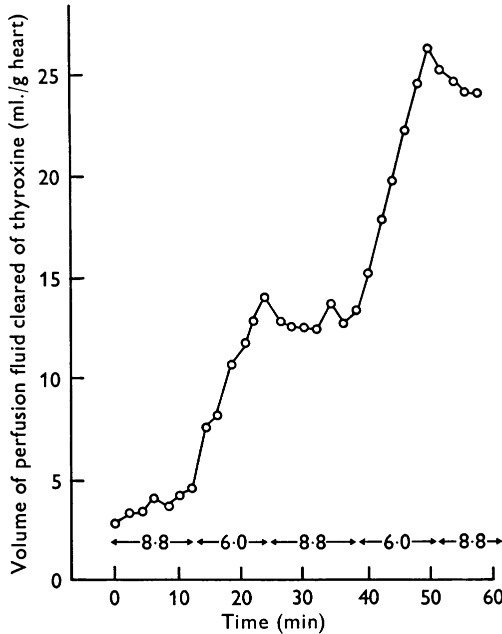


Fig. 5. The cumulative uptake of thyroxine by a rat heart perfused with Krebs–Ringer phosphate solution. The pH was switched between 6·0 and 8·8 during the course of the experiment. The uptake is expressed as the volume of perfusion fluid cleared of the hormone per gram of heart tissue. Thyroxine concentration 1×10^{-3} μg/ml.

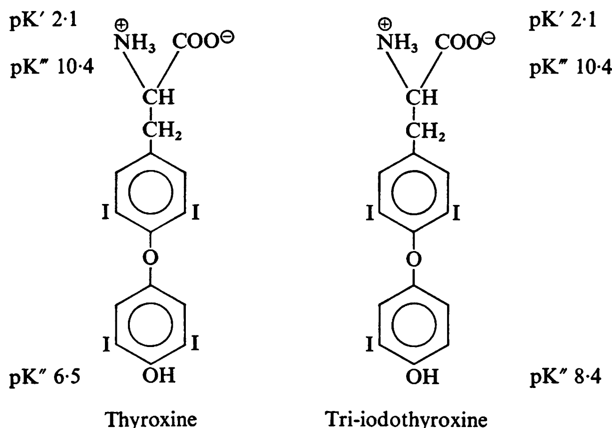
TABLE 1. The amount of thyroxine and tri-iodothyronine taken up by rat hearts during 60 min perfusion with Krebs–Ringer phosphate solution at pH 6·0 and 8·8. The uptake is expressed as the volume of perfusion fluid cleared of the hormone per gram of heart tissue. There were five hearts in each group and the values are given as means ± S.E. of mean. Hormone concentration 1×10^{-3} μg/ml.

	Thyroxine	Tri-iodothyronine
pH 6·0	52·1 ± 3·5	64·0 ± 6·2
pH 8·8	9·3 ± 0·8	37·2 ± 3·4

than the binding of tri-iodothyronine, such that, at pH 6, the uptake of the hormones was fairly similar, although the uptake of tri-iodothyronine was still significantly higher than the uptake of thyroxine. At pH 8·8 on the other hand very much more tri-iodothyronine was taken up than thyroxine.

DISCUSSION

Simple phenolic groups have a pK of about 10. Tri-iodothyronine has a single iodine atom at the *ortho* position giving its phenolic group a pK of 8·4; thyroxine has two iodine atoms giving a pK of 6·5. This difference in the acid strength of the phenolic groups is probably responsible for the difference in membrane binding between the hormones at various pH. The binding of thyroxine falls off sharply when the pH of the bulk aqueous phase is about 8·0. The pH at the membrane surface will be lower than this, probably about 6·5, i.e. near the pK for the phenolic group of the hormone. A similar argument can be made for tri-iodothyronine. The binding of thyroxine to membranes appears therefore to be associated with lack of charge on this phenolic group. In this condition all of the aromatic portion of the molecule will be uncharged and therefore hydrophobic. 'Hydrophobic bonding' (Chapman, 1969) will tend to attract this part of the molecule into the lipid interior of the membrane whereas the hydrophilic side chain will tend to lie in the outer watery layer. The stability of thyroxine adsorbed in this way at an oil–water interface may constitute the main force binding the hormone to these membranes.



It has been proposed that thyroxine-binding sites on cells must compete with the binding sites on plasma proteins for the available, free thyroxine in plasma (Hillier, 1970). The binding of thyroxine by plasma proteins is

very rapid (Hillier, 1970); it follows therefore that the tissue thyroxine-binding reaction must also be rapid. Interaction of thyroxine with the surface membrane of cells probably constitutes the initial step in this binding process; the trapped hormone could then pass fairly readily through the membrane to further membranes or binding sites within the cell. The high permeability of membranes to thyroxine probably indicates that the concentration of free thyroxine is fairly similar throughout the plasma and the tissues.

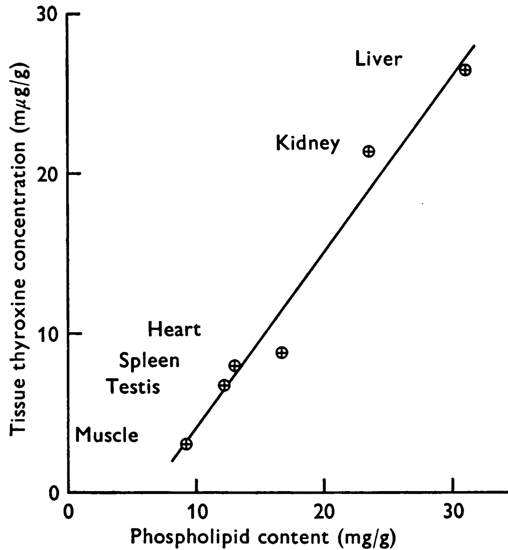


Fig. 6. The concentration of thyroxine in various rat tissues (from Albright *et al.* 1965) plotted against their phospholipid content (from Dawson, 1957).

Experiments with perfused hearts and lipid extracts of liver suggest that a large proportion of the thyroxine present in tissues could be bound to lipid membranes. Using a 'partition coefficient' of 10^4 it is possible to calculate that there is more than enough phospholipid in tissues to account for all of the thyroxine trapped by the heart *in vivo* although perhaps not by the liver. In view of the doubtful relevance of a partition coefficient in this context and the number of assumptions necessarily made in the calculations, the results cannot be very persuasive. There is however a correlation between the phospholipid content of tissues (an index of their membrane density) and their ability to bind thyroxine *in vivo* (Fig. 6; phospholipid concentrations are taken from a review by Dawson (1957) and thyroxine concentrations from Albright, Heninger & Larson, 1965). Further, Schwartz, Bernstein & Oppenheimer (1969) noted that more than

half of the thyroxine in liver cells is bound by the endoplasmic reticulum, a structure composed mainly of phospholipid membranes, and that drugs which cause its proliferation also increase the thyroxine-binding power of the tissue. They suggested that thyroxine was binding to specific proteins associated with the membranes although it is possible that the hormone was simply binding to the phospholipid.

Thyroid hormones will tend to aggregate at membrane surfaces in very much higher concentration than will be present in free solution. This rather non-specific concentrating effect may serve as the initial step in transferring the hormone to its sites of action or metabolism. The tissue 'receptor' for thyroxine is normally supposed to react with the thyroxine present in free solution (Tata, 1964) and on this hypothesis the tissue thyroxine-binding reaction is largely irrelevant, in that it has little effect on the free thyroxine concentration (since the hormone readily penetrates cell membranes). An alternative hypothesis is that the thyroxine receptor and the enzymes concerned with its metabolism are membrane bound, so that the amount of the hormone available to them is not determined by the level of free thyroxine but by the local concentration at the membrane surface.

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